Award Number: W81XWH-12-1-0274

TITLE: Diagnosis and Treatment of Heterotopic Ossification

PRINCIPAL INVESTIGATOR: Dr. Alan R. Davis

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, TX 77030

REPORT DATE: October 2015

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release; Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2015	Annual	30Sep2014 - 29Sep2015

4. TITLE AND SUBTITLE

Diagnosis and Treatment of Heterotopic Ossification

5a. CONTRACT NUMBER

W81XWH-12-1-0274

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Dr. Alan R. Davis, and Dr. Elizabeth A. Olmsted-Davis

E-Mail: ardavis@bcm.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Baylor College of Medicine
Houston, TX 77030

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

We recently developed a model of heterotopic ossification (HO) that suggests blocking the initial response of the nerve could ultimately block this devastating problem before it occurs. Studies proposed in this application to identify and isolate osterix+ cells have led us to identify a second tentative progenitor within peripheral nerves. These cells reside in the endoneurial compartment and express Claudin 5, PDGFRalpha, and osterix but are negative for the Schwann cell marker P75 and perineurial marker Claudin 1. We have isolated the Claudin 5+, PDGFR+ and negative populations and confirmed that 100% of the osterix expression co-purified with the claudin 5+ population. Claudin 5 is present on specialized endothelial cells within the endoneurium and is responsible for forming the tight junction of blood nerve interface. We are further characterizing these cells for expression of endothelial markers. We also looked at the expression of Wnt1, a marker of neural crest stem cells. Our preliminary data suggests that these cells undergo significant expansion after induction of HO; however, they remained localized within the peripheral nerves. However, 100% of the Wnt1+ cells appeared to be also claudin 5+ suggesting that the Wnt1 cells may be precursors to a more differentiated progenitors. We are currently following up on these and other experiments proposed in the application.

15. SUBJECT TERMS
BMP2, Heterotopic ossification, endoneurial cells.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	21	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

	Page
Introduction…….……..…..	7
Body……..	4
Key Research Accomplishments…….……..	20
Reportable Outcomes……	21
Conclusion……	21
References…….	21

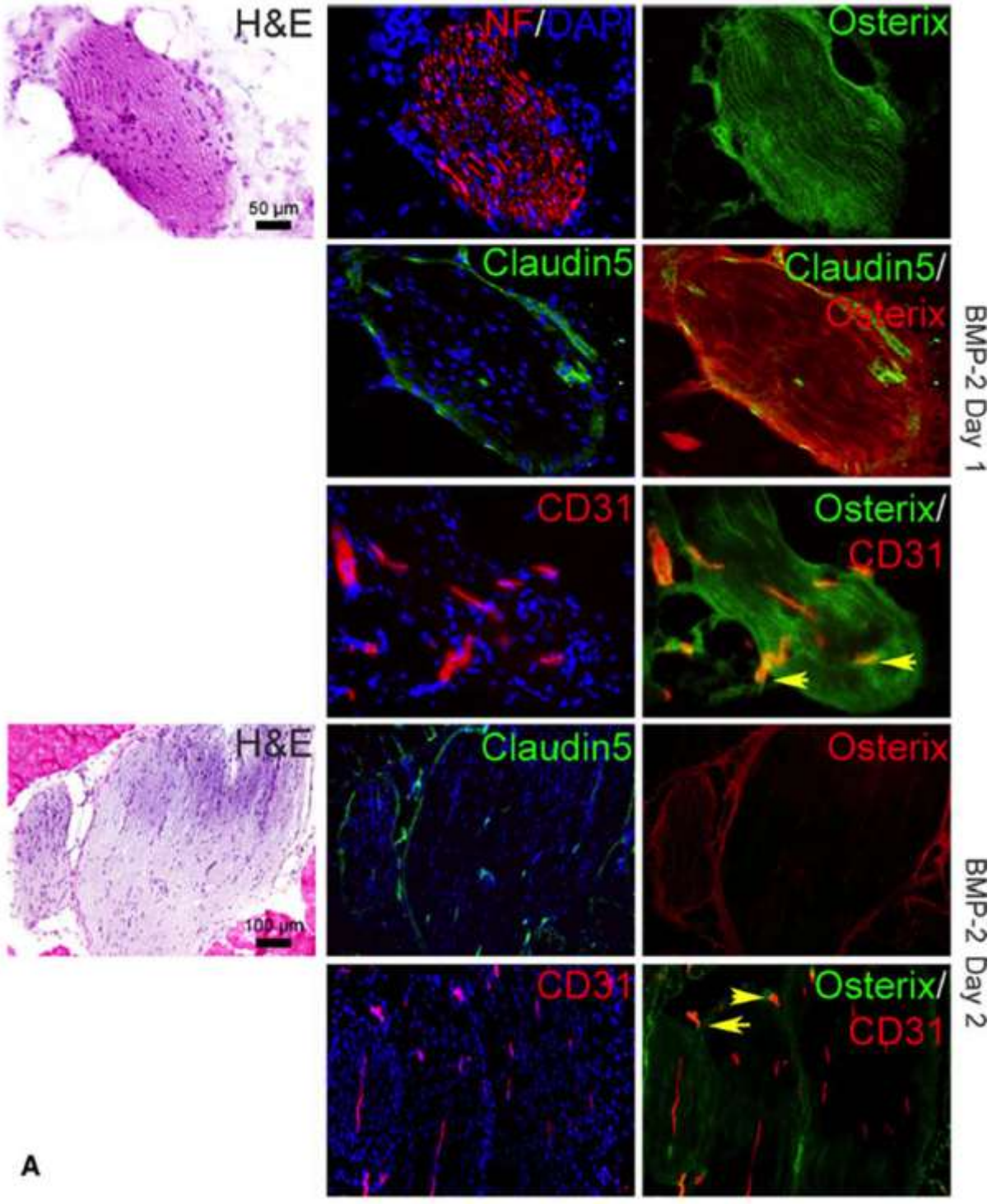
INTRODUCTION:

Heterotopic ossification (HO), the formation of bone in the muscle or other soft tissue, or any non-skeletal site can causes severe problems of pain and disability. It often requires the patient to undergo additional surgery. A particularly frustrating problem in amputees is the growth of bone within the amputation stump, making prosthesis wear difficult or impossible. Such heterotopic bone also develops spontaneously near the joints in many patients with an injured spinal cord. Tentative inhibitors, such as low dose radiation that have some efficacy in preventing HO in patients at high risk, cannot be implemented in the majority of cases. Thus there are currently no available efficacious treatments. Although the incidence of HO in the general populations is fairly low, approximately 11% of all musculoskeletal injuries, it is a significant problem within the military population where the incidence is approximately 60-70% of all traumatic injuries [Alfieri et al., 2012]. Here we present data we have recently published [Lazard et al., 2015] that indicate that in actuality the primary source of HO is the peripheral nervous system (PNS). We recently identified one of the earliest steps in HO is the remodeling of the nerve structure through a key process induced by BMP2. Preliminary results (not covered in this report) indicate the cell that is the target of BMP2 signaling. Surprisingly, we have found this cell to be, at early times after induction of HO, directly adjacent to the perineurium of peripheral nerves and to carry many markers of the astrocyte in the CNS. We speculate therefore that the first step in BMP2 signaling in HO is the generation or modulation of an electrical signal within the nerve, which is an under-appreciated but important property of the astrocytes. We hypothesize that the regulation of heterotopic ossification may be induced through neurogenic inflammation and subsequent peripheral nerve electrical signaling, which leads to remodeling of the nerve and release of osteoprogenitors. Here we propose that remodeling of the nerve itself, leads to the release of stem/progenitors from the nerve, which contribute to the structures that make up bone, including, but not restricted to, osteoblasts.

In this application we proposed to characterize these progenitors, demonstrate their functional role in HO, and utilize the mechanism of this process for the development of a blood test for early detection. Knowledge of mechanism also specifies molecular targets for design of agents to treat HO. Although HO seems like a disease occurring mostly in the military from blast injuries, we have also recently shown that it occurs in the vessels of the heart [Salisbury et al., 2012a]. This makes understanding the mechanism of HO even more important, since heart disease is still a major kill in the U.S. where greater than 1 in 3 have cardiovascular disease [Mozaffarian et al., 2015]. Additionally, we speculate that HO may have skeletal manifestations beyond ectopic bone formation. In our mouse model of HO we have noted an inverse correlation between the amount of heterotopic bone formed and the state of skeletal bone. For instance in the Misty mouse, which lacks a functional protein encoding Dock 7, important for brown adipocyte biogenesis, heterotopic bone is present at high levels, while skeletal bone is severely osteoporotic [Olmsted-Davis et al., 2007]. Therefore it is conceivable that, the presence of HO may not only cause the problem of ectopic bone, but also may make repair of skeletal bone much more difficult.

Task 1: To isolate and characterize the nerve stem/progenitor population: Since their original description in 1999 [Pittenger et al., 1999] bone marrow mesenchymal stem cells have

been thought of as the progenitor for osteoblasts during HO [Pittenger et al., 1999]; however, recent work suggests that there may be a local stem/progenitor cell [Wosczyna et al., 2012] [Lounev et al., 2009]. Using our model of HO, we recently identified the rapid expansion of a progenitor within the endoneurium of peripheral nerves that expressed the stem cell markers nanog and Klf4, as well as osterix, a transcription factor that regulates osteogenesis. The endoneurium of peripheral nerves houses the axons and Schwann cells. This compartment is separated from the external environment by the perineurium, which functions, along with the endoneurial vasculature, as a barrier [Yosef et al., 2010]. We next attempted to determine whether osterix expression within the endoneurium was associated with one of the known cell populations that reside in this region. Immunostaining the tissues for the Schwann cell markers (proteolipid protein 1 and nerve growth factor receptor, P75) showed that they did not coincide (data not shown). Recently Yosef et al [Yosef and Ubogu] reported the unique expression of the tight junction molecule, claudin 5, on specialized endoneurial endothelial cells (EECs) and suggested it plays a critical role in regulating the blood-nerve interface similar to that observed in the brain where claudin 5 (-/-) mice do not survive because of the lack of an effective blood brain barrier [Nitta et al., 2003]. Furthermore even though many ECs express claudin 5 under diverse circumstances [Nitta et al.], it is only expressed in peripheral nerves in the mouse hind-limb tissues prior to induction of HO (Figure 1). Immunohistochemical staining for claudin 5 expression in mouse hind-limb shows the presence of a handful of cells within the endoneurium of peripheral nerves (as detected by neurofilament H) (Figure 1, panel A), but absent from any other tissue structures including the normal blood vasculature (Figure 1, panel A). However four days after induction of HO, these claudin 5+ cells can now be seen both inside and outside of the nerve structures, and in between muscle fibers, which reside between peripheral nerves and the site of new bone formation (Figure 1, panel B).


H&E
NF/DAPI
Osterix
50 μm
Claudin5
Claudin5/
Osterix
CD31
Osterix/
CD31
BMP-2 Day 1
H&E
Claudin5
Osterix
100 μm
CD31
Osterix/
CD31
BMP-2 Day 2
A

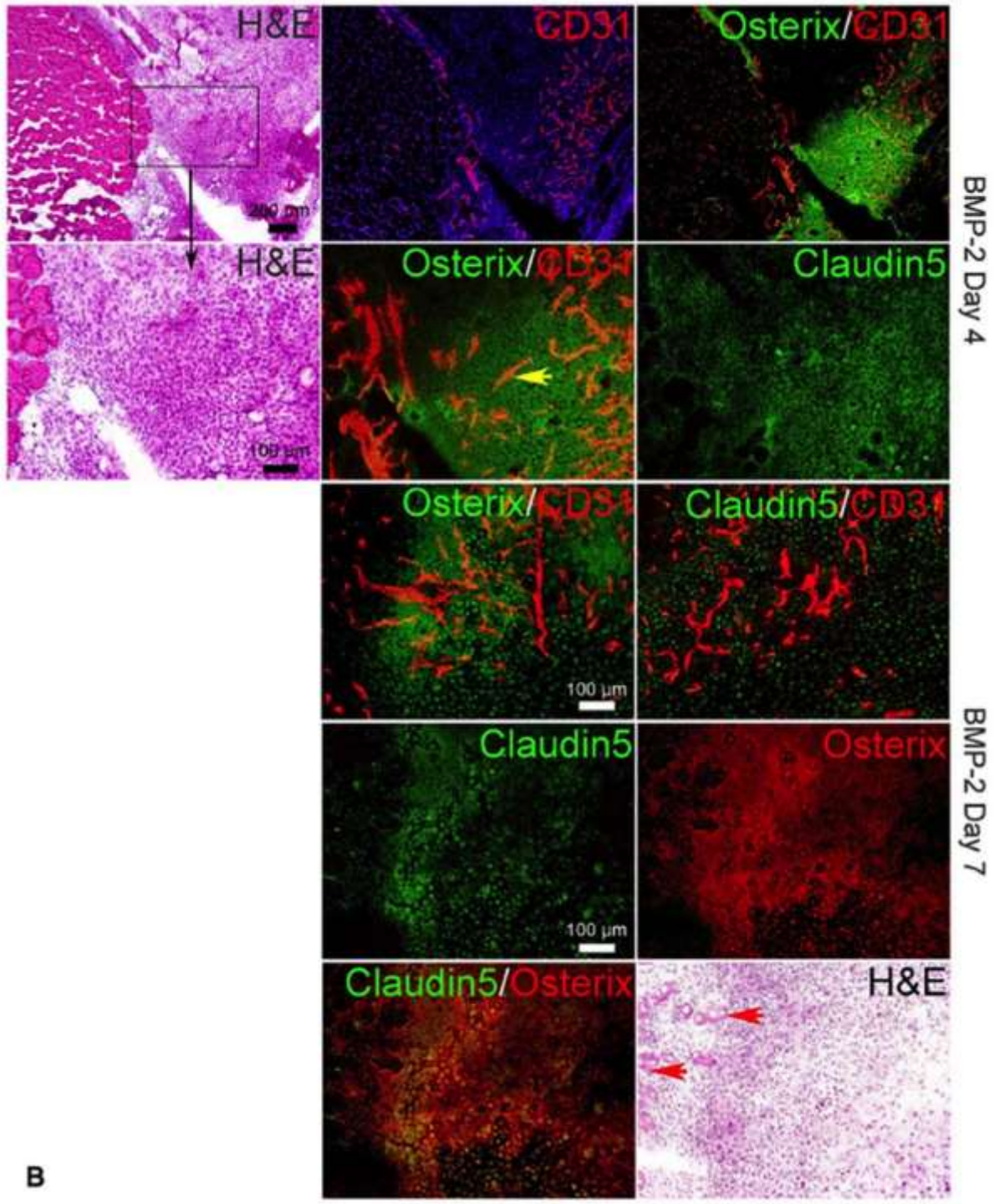

H&E
CD31
Osterix/CD31
H&E
Osterix/CD31
Claudin5
Osterix/CD31
Claudin5/CD31
100 μm
Claudin5
Osterix
100 μm
Claudin5/Osterix
H&E
BMP-2 Day 4
BMP-2 Day 7
B

Fig. 1A–B (**A**) Osterix expression begins in the endoneurium of peripheral nerves. C57BL/6 mice (n = 8) were injected with BMP-2-producing cells and four mice each were euthanized on Days 1 and 2. Frozen sections were prepared and immunostained for neurofilament heavy chain (NF), CD31, or osterix. DAPI is blue. H and E sections were stained with hematoxylin and eosin (Stain, NF; original magnification, × 20; CD31; original magnification, × 20; osterix, original magnification, × 20). Some of the vessels within the endoneurium expressed both osterix and CD31 (yellow arrows). (**B**) Expression of osterix, claudin 5, and CD31 at later times after BMP-2 induction (Stain, osterix; original magnification, × 20; claudin 5; original magnification, × 20; CD31, original magnification, × 20). C57BL/6 mice (n = 16) were injected with BMP-2-producing cells and eight mice each were euthanized at either Day 4 or 7. Frozen sections were prepared and immunostained for CD31, claudin 5, or osterix (Stain, osterix; original magnification, × 20; claudin 5; original magnification, × 20; CD31, original magnification, × 20). Some of the vessels within the endoneurium expressed both osterix and CD31 (yellow arrows). Some of the vessels within the endoneurium expressed both osterix and CD31 (yellow arrows). H&E = hematoxylin and eosin.

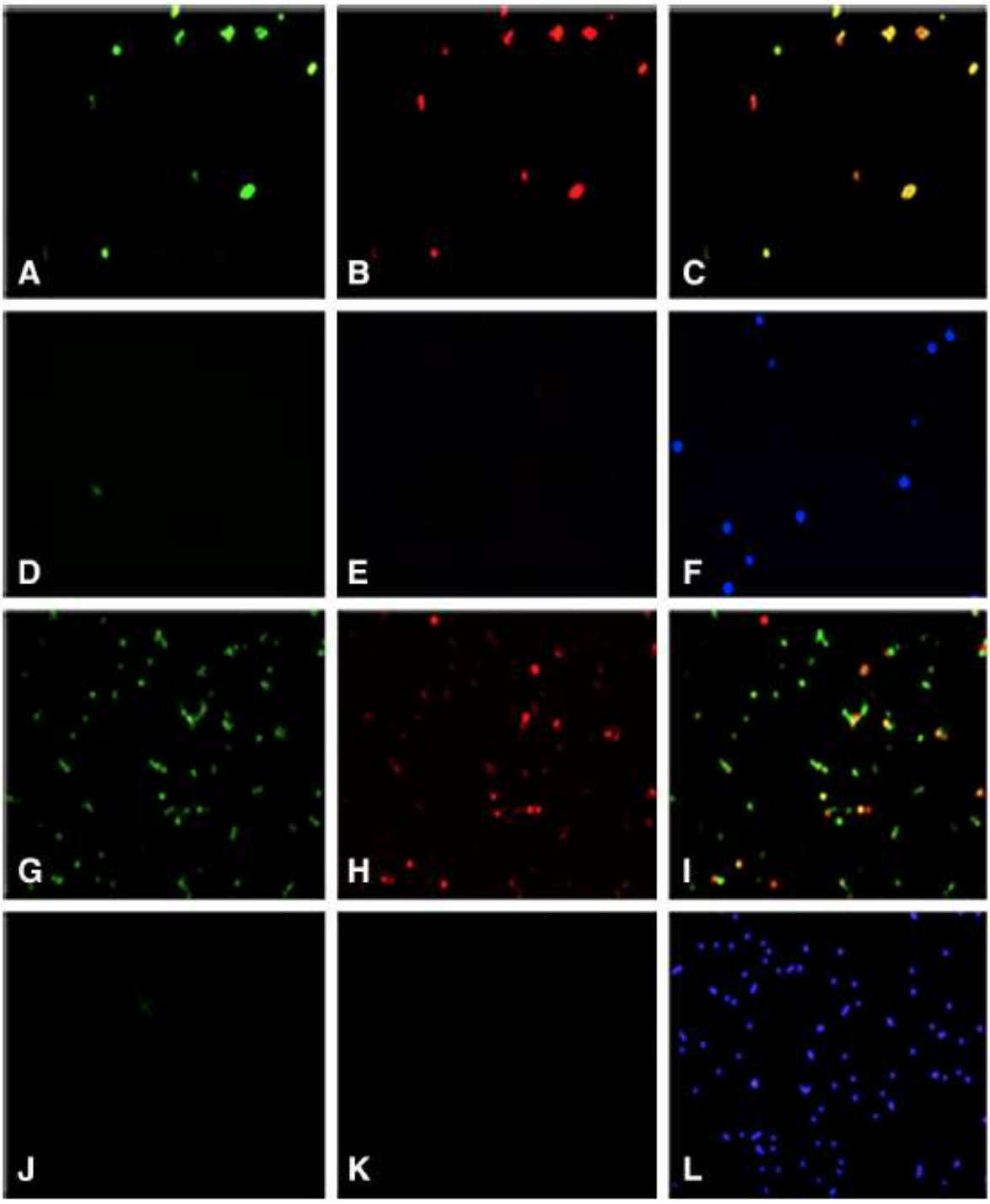


Fig. 2A–L Claudin 5-positive cells express osteogenic markers. The claudin 5^+ population (green) was isolated from a FACS of cells isolated from muscle 4 days after BMP-2 induction. These isolated cells were subjected to cytospin and the slides were then probed with antibodies for claudin 5 (green) and osterix (red). (**A–C**) One field obtained from the claudin 5^+ population with **C** being the merger of **A** and **B**; (**D–F**) one field of the cytospin of a claudin 5^- population obtained from the same mouse that was stained with antibodies against claudin 5 (green) and osterix (red) as well as DAPI (**F**). In the claudin 5^+ cell population, osterix-positive cells were found to be 75% ± 3%. In **G-I** and **J-L**, respectively, we show the cytospin patterns of the claudin 5-positive and -negative populations of another mouse after staining for claudin 5 (green) and *dlx* 5 (red).

We next determined whether these cells were co-localizing with the osterix+ cells in the nerve. Osterix appeared to co-localize to the nucleus of claudin 5+ cells, but from these initial immunostains, we cannot conclude that these cells are migrating away from the nerve towards HO, but the expression pattern involves the area that is between the reacting nerves and new bone, which is highly suggestive that the nerve structure is involved in this process.

We next quantified the number the claudin 5+ cells through fluorescence activated cell sorting (FACS). In these experiments, soft tissues were isolated at time 0, 2, and 4 days after induction of HO, collagenase digested, and cells subjected to cell sorting. The results confirm that claudin 5+ cells increase almost 10-fold as early as 2 days after induction of HO, but then decline somewhat by day 4, although still significantly elevated over time 0 (or control). This may reflect the initial expansion of these EECs and differentiation to osteoblasts. We next confirmed the co-expression of claudin 5 and osterix, by immunostaining the isolated cells. Both the claudin+ and – populations (green) were collected and cytospun onto slides, which were further immunostained for osterix (red) (Figure 2). Cells were counterstained with DAPI (blue). The majority (approximately 75%) of the claudin 5+ population also expressed osterix (panel B and E). As seen in panel C, and F, when we overlayed the images, we observed some osterix+ cells that were very weakly positive for claudin 5 and some claudin 5+ cells that were not expressing osterix, suggesting that the cells may be down regulating claudin 5 as they up-regulate osterix. The claudin 5- cells, figure 5, panels G, H and I, had similar numbers of cells as the positive population (Panel I), but we observed no positive staining for osterix, panel H and I.

We next looked at the expression of PDGFRα in these tissues. Previous reports suggest that PDGFRα is expressed only in primitive endoderm in early embryogenesis and then during organogenesis, in regions of epithelial and mesenchymal interaction, such as the tooth bud and bronchi and mesodermal derivatives, the lens, apical ectodermal ridge, glial precursors, cardiac valves, and choroid plexus [Lazard et al.]. FACS revealed that 95% of the claudin 5+ cells also expressed PDGFRα, and 75% of the PDGFRalpha cells were double positive. Further, there was a significant increase in these cells during HO (data not shown but see [Lazard et al.]), suggesting that our tentative endoneurial endothelial-like progenitors may be similar to those osteogenic precursors previously described [Wosczyna et al., 2012].

Interestingly, previous reports demonstrated that the potential endothelial-like progenitor does not arise from blood vasculature within the muscle fibers [Wosczyna et al.]. Thus we hypothesized that it is highly likely that this cell is derived from the nerve. However, suppression of nerve remodeling did not result in suppression of their expansion. In these studies, we suppressed nerve remodeling via neuroinflammation through delivery of cromolyn. FACS analysis of the whole tissues isolated 4 days after the induction of HO, in the presence of cromolyn or vehicle, showed a similar expansion of these cells (data not shown). However, it is

unclear whether the claudin 5+ cell expansion in the presence of cromolyn, shows the same pattern as in Figure 1, or whether they are entirely within peripheral nerves, as previously reported for osterix [Salisbury et al., 2011].

Since we previously showed that the osterix+ cells also expressed stem cell markers (nanog and Klf4), we next determined whether these cells also expressed Wnt1. Wnt1 is critical in the induction and migration of neural crest stem cells in the embryo [Zhang et al.] [Medici et al.]. In addition, it has been suggested as a key factor in the epithelial to mesenchymal transition [Gonzalez and Medici, 2014] as well as a surprisingly important factor in angiogenesis [Gherghe et al., 2011] Since endothelial progenitors were recently reported to undergo an EMT in HO we questioned whether these EECs could be derived from a Wnt1+ neural crest progenitor.

We also analyzed the claudin 5+ cells for the embryonic markers nanog and Wnt1 as well as the endothelial marker Tie2. Results show that each of these markers is present in the claudin 5+ but not the claudin 5- negative cell populations. We consider it likely that the claudin 5+ endoneurial endothelial-like cells is indeed a progenitor for the osteoblast. Indeed Wnt1 [Wang et al., 2014], nanog [Arpornmaeklong et al., 2009]and Tie2 [Medici et al., 2010] have been reported by others to be expressed in osteoprogenitors using various methodologies.

Task 2: To identify the functional contribution of these stem/progenitors to heterotopic ossification.

To test whether the tentative osterix+ cells are contributing to osteoblast populations in HO, we employed a lineage-tracing mouse (Ert Wnt1Cre x R26R td tomato red), which permanently activates td tomato red expression when the Wnt1 promoter is activated in the presence of tamoxifen. Our first experiments, however, were performed in a similar mouse that permanently express YFP instead of tomato red. These mice were pre-treated with tamoxifen or vehicle, and then tissues isolated 4 days after induction of HO to determine if the claudin 5+ cells co-localized with YFP label. YFP expression was observed within the nerves 4 days after induction of HO, but absent in nerves in the control, suggesting that Wnt1 expression had been upregulated by BMP2. However we did not observe many cells outside the nerve, suggesting that the Wnt1 labeled cells may be repopulating tentative nerve progenitors, rather than co-localizing with claudin 5+ cells. We also performed preliminary FACS analysis of cells isolated from these animals, and found that only a minor percentage of the claudin 5+ cells co-expressed Wnt1 (data not shown). Thus the expansion of Wnt1+ cells suggests that they may be an earlier stem/progenitor than the claudin 5+ osterix+ cells migrating from the nerve.

Therefore to confirm this hypothesis and demonstrate a nerve origin of the claudin 5+ cells, we tried a prolonged treatment with tamoxifen to "chase" intermediate progenitors and allow usage of early Wnt 1 positive ones. To do this the mice will be exposed to tamoxifen at the time of weaning, so as to provide additional time for cellular attrition, and labeling of more differentiated cells derived from the Wnt1+ cell. Secondly, we will induce neuroinflammation in the absence of BMP2, to allow for nerve remodeling, and repopulation of the progenitors from the stem cells expressing YFP. This will be done several weeks prior to inducing HO and then we will track the YFP lineage marker to determine if we now see the YFP within osteoblast populations.

We also proposed in this aim to test the functionality of these osterix+ cells through transplantation of a portion of the nerve from an osteocalcin-cre x R26R-YFP mouse into a wild type animal, and then follow whether we observe YFP+ cells within bone. Since the surgical defect in the nerve created through the transplantation experiment will potentially result in an altered environment, we propose to take a different approach to definitively test whether these osterix+ cells are contributing directly to osteoblasts in HO. Our proposed approach will be to cross mice that have the endogenous osterix gene flanked with lox P sites, so that in the presence of cre, osterix expression will be ablated. We will therefore cross the tamoxifen-regulated Wnt1-Cre and/or PDGFRα-Cre into these mice, so that only the cells expressing either wnt1 or PDGFRα will be ablated for osterix. Therefore, we can induce HO in these mice, and characterize the resultant bone formation as to the number of osteoblasts, bone volume, density, and other parameters. We predict that since osterix is a master transcription factor for osteoblasts, that selective ablation in these cells, will disrupt bone formation during HO, if they are directly utilized as progenitors. Our collaborator Dr. Benoit de Crombrugghe has agreed to provide us his previously generated and characterized floxed osterix mice.

Finally, we also made the exact same reporter mouse (Ert Wnt1-Cre x R26R td Tomato Red), but substituting td Tomato Red for YFP as the reporter. Surprisingly, this mouse was far more robust in reporter expression with far less background. It has been known for some time that the red and infra-red wavelengths give far less background even though signal strength is somewhat compromised. Overall the signal/noise ratio is much lower for td Tomato Red. This is strikingly exemplified in Figure 3. In these mice tamoxifen was injected two days before BMP2-producing cells, and the mice were euthanized two days later. If either tamoxifen or BMP2-producing cells were omitted there was virtually no red (data not shown).

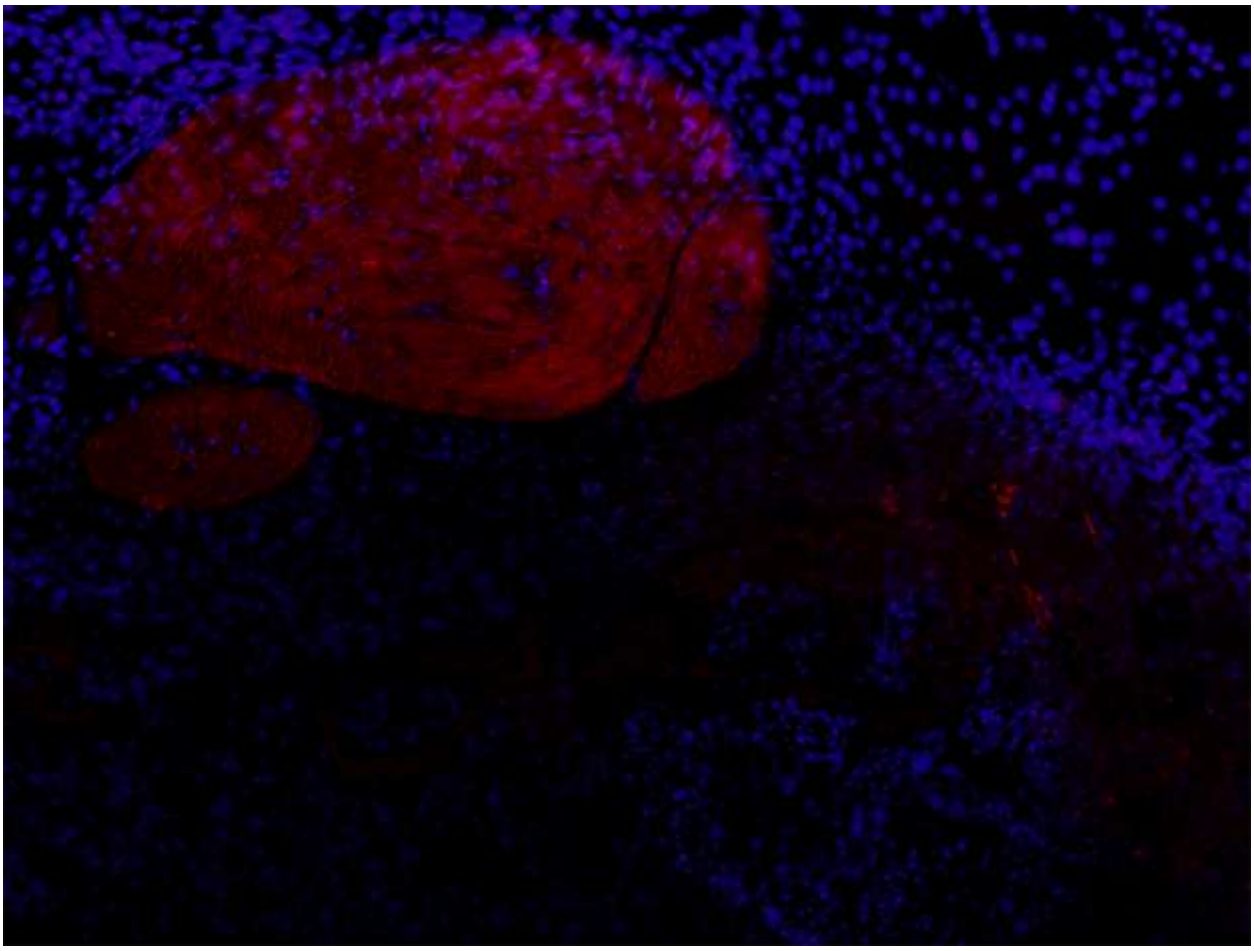


Figure 3. Peripheral nerves are positive for td Tomato red signal after one day induction with BMP2. Ert-Wnt1-Cre/R26R td Tomato red mice were injected with tamoxifen on day -2, -1, and 0 and on day 0 were also injected with BMP2-producing cells. On the first day after BMP2 induction the mice were euthanized, the hind limbs were harvested in sucrose, quick frozen, and 4 μm sections prepared.

When BMP2-producing cells and tamoxifen were injected on day 0 and mice were euthanized on day 3, one can see that quite a few cells are labeled with an antibody to claudin 5 that are also positive for td Tomato red (data not shown). Also the presence of red fluorescence is totally dependent upon the addition of both tamoxifen and BMP2.

When BMP2-producing cells and tamoxifen were injected on day 0 and mice were euthanized on day 7, one can see that many of the osteoblasts at the site of bone formation exhibit red fluorescence (Figure 4). To show that these red cells were indeed osteoblasts on the surface of bone, the slide was also photographed uner polarized light (Figure 4). This is a conformation of the studies that we published concluding that osteoblasts in HO originate from peripheral nerves.

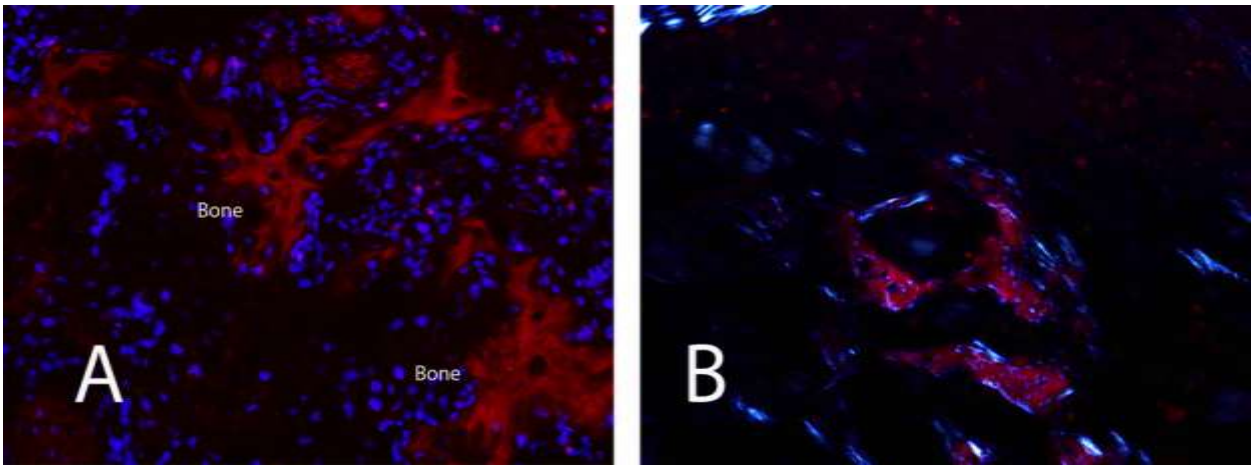


Figure 4. Osteoblasts are derived from peripheral nerves. Ert-Wnt1-Cre/R26R td Tomato red mice were injected with tamoxifen on days -2, -1, and 0 and on day 0 were injected with BMP2-producing cells. On the seventh day after BMP2 induction the mice were euthanized, the hind limbs were harvested in sucrose, quick frozen, and 4 μm sections prepared.

To further confirm that these were osteoblasts that exhibited red fluorescence, Ert-Wnt1-Cre/R26R td Tomato red mice were treated with tamoxifen on days -2, -1, and 0 and on day 0 were also injected with BMP2-producing cells. The mice were euthanized on day 7 and the muscle around the site of injection was isolated, homogenized, and treated with collagenase. This digest was subjected to FACS with an antibody against mouse claudin 5 conjugated to Alexa fluor 488. The positive cells were collected and subjected to cytospin on a microscope slide and then analyzed under a fluorescent microscope. The result shown in Figure 5 confirms that most of the claudin 5 positive cells were also positive for td Tomato red indicating that they were originally derived from neural cells.

Finally, to quantitate the type of cells that are induced in The ErtWnt1 Cre x R26R td tomato red mouse, one group of these mice (n=6) received tamoxifen at days -2, -1, and 0, also receiving 5 x 10^6 BMP2-producing cells on day 0. Another group (n=6) received only the tamoxifen at days -2, -1, and 0. Finally , a third group (n=6) received only the BMP2-producing cells on day 0. Mice were euthanized at day 4 and the muscle around the site of injection was isolated, homogenized, treated with collagenase and filtered before reacting the isolated cells with an antibody against mouse claudin 5 followed by an anti mouse antibody labeled with Alexa fluor

488. FACS analysis was carried out on a Becton Dickenson FACSARIA.

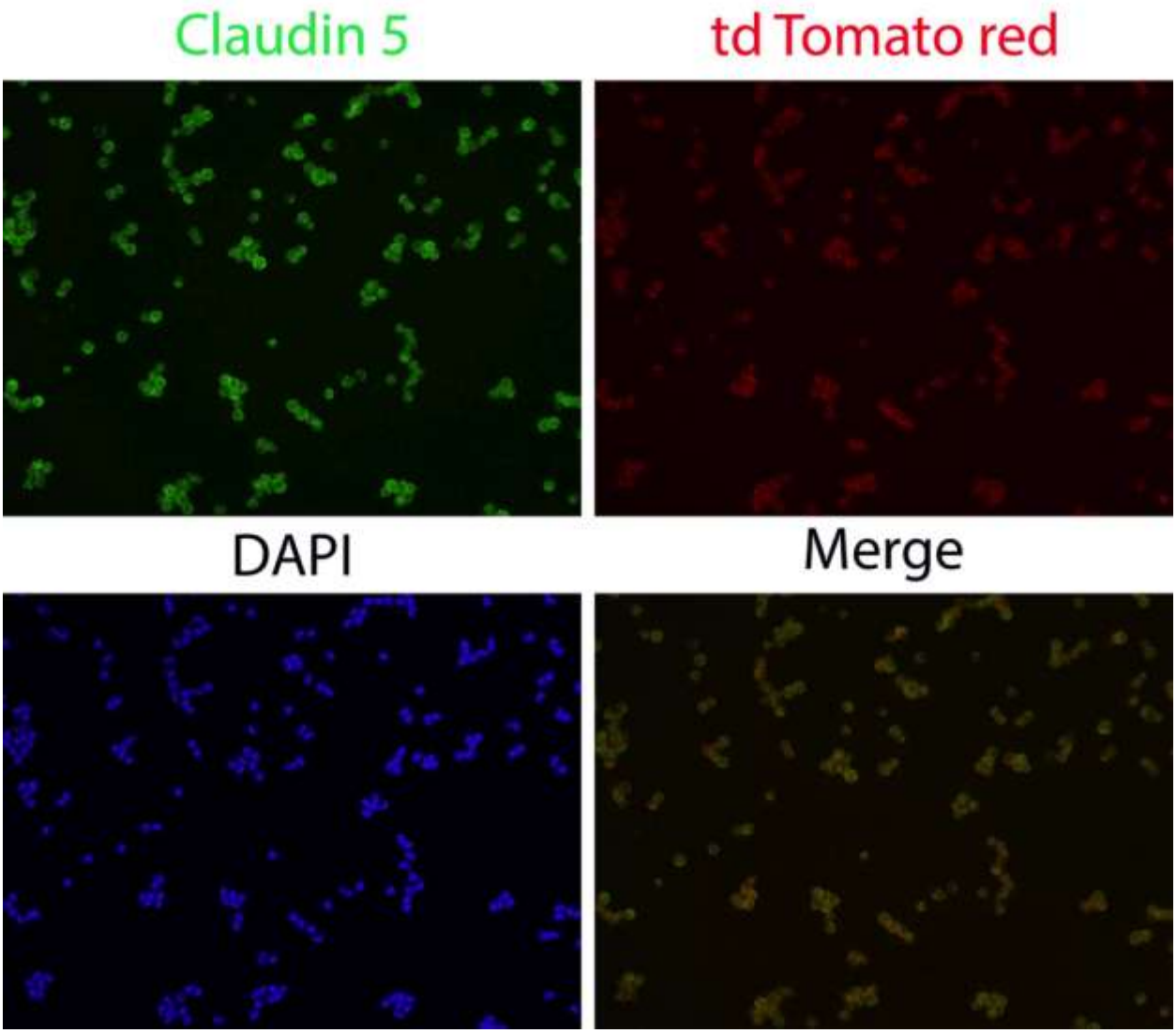


Figure 5. Osteoblasts formed during HO are derived from the nerve. Ert-Wnt1-Cre/R26R td Tomato red mice were injected with tamoxifen on days -2, -1, and 0 and were also injected with BMP2-producing cells on day 0. After seven days the mice were euthanized, the muscle around the site of injection was homogenized, digested with collagenase, and reacted with an antibody to mouse claudin 5 and then with an anti-mouse antibody conjugated to Alexa fluor 488. Cells were then subjected to cytospin and the slides coversliped with a DAPI-containing reagent.

The data (Figure 6) clearly shows that both tamoxifen- and BMP2-induction must take place in order to induce claudin 5 expression in the tissue. The data also indicates that claudin 5 expression is important at 4 days after BMP2 induction since it is specifically induced and present in both the TR+claudin5+ and TR-claudin5+ cell populations. We have shown previously that claudin 5 is present on both osteoprogenitors and osteoblasts during HO [Lazard et al., 2015], and we speculate that one of the reasons for this is that this key tight junction molecule, found exclusively in a type of endothelial cell whose relationship to other endothelial

cells has not been defined [Morita et al., 1999], is also a critical component of the blood-brain barrier [Argaw et al., 2009], is expressed as part of a mechanism to aid osteoprogenitors in crossing into the endoneurial vessels from the neural endoneurium so that they can flow through vessels to the site of bone formation [Lazard et al., 2015].

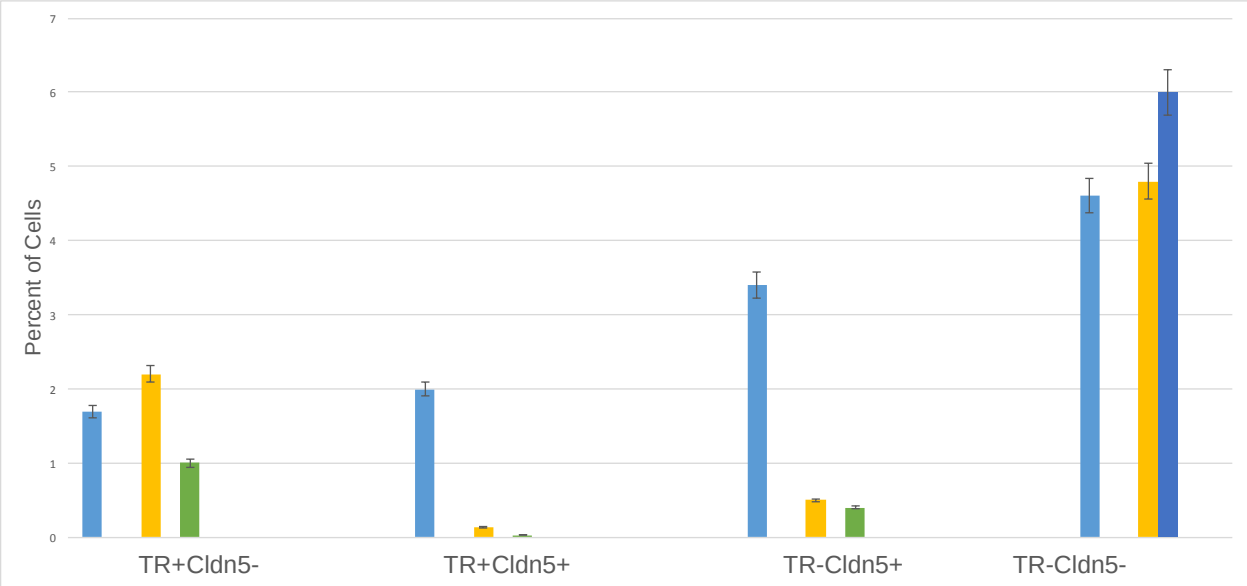


Figure 6. Changes in key cell populations after simultaneous BMP2 and tamoxifen induction of the ErtWnt1 Cre x R26R td Tomato red mouse. These mice were induced simultaneously with BMP2 and tamoxifen (n=6, blue) or with only BMP2 (n=6, yellow) or with only tamoxifen (n=6, green).

An explanation that is consistent with our findings is that there is not only a progenitor for the osteoblast in peripheral nerves, but for other cells that are involved in HO as well. To address this we looked at other cell types including transient brown adipocytes and chondrocytes for the presence of the td Tomato Red marker after induction with BMP2. As shown in Figure 7, when the Ert Wnt1Cre x R26R td Tomato red mouse is induced with tamoxifen on days -2,-1, and 0 and BMP2-producing cells are injected on day 0, 5 days after BMP2-induction sections were stained for the transient brown adipocyte marker ADRB3. As expected there are many cells that are positive for the tomato red marker, but are not positive for ADRB3. However, there are a

number of cells positive for both ADRB3 and td Tomato red, indicating that transient brown fat may be derived from the same progenitor cell within the peripheral nerve as the osteoprogenitor.

This is consistent with the fact that transient brown fat is derived from the perineurial region of peripheral nerves [Salisbury et al., 2012b].

We next examined pre-chondrocytes using the Sox9 marker, which is a well-established marker for these cells [Piera-Velazquez et al., 2007]. We again found significant overlap of the pattern of Sox 9 labeling and td Tomato red, indicating that these cells too are most likely derived from a common progenitor in the peripheral nerve.

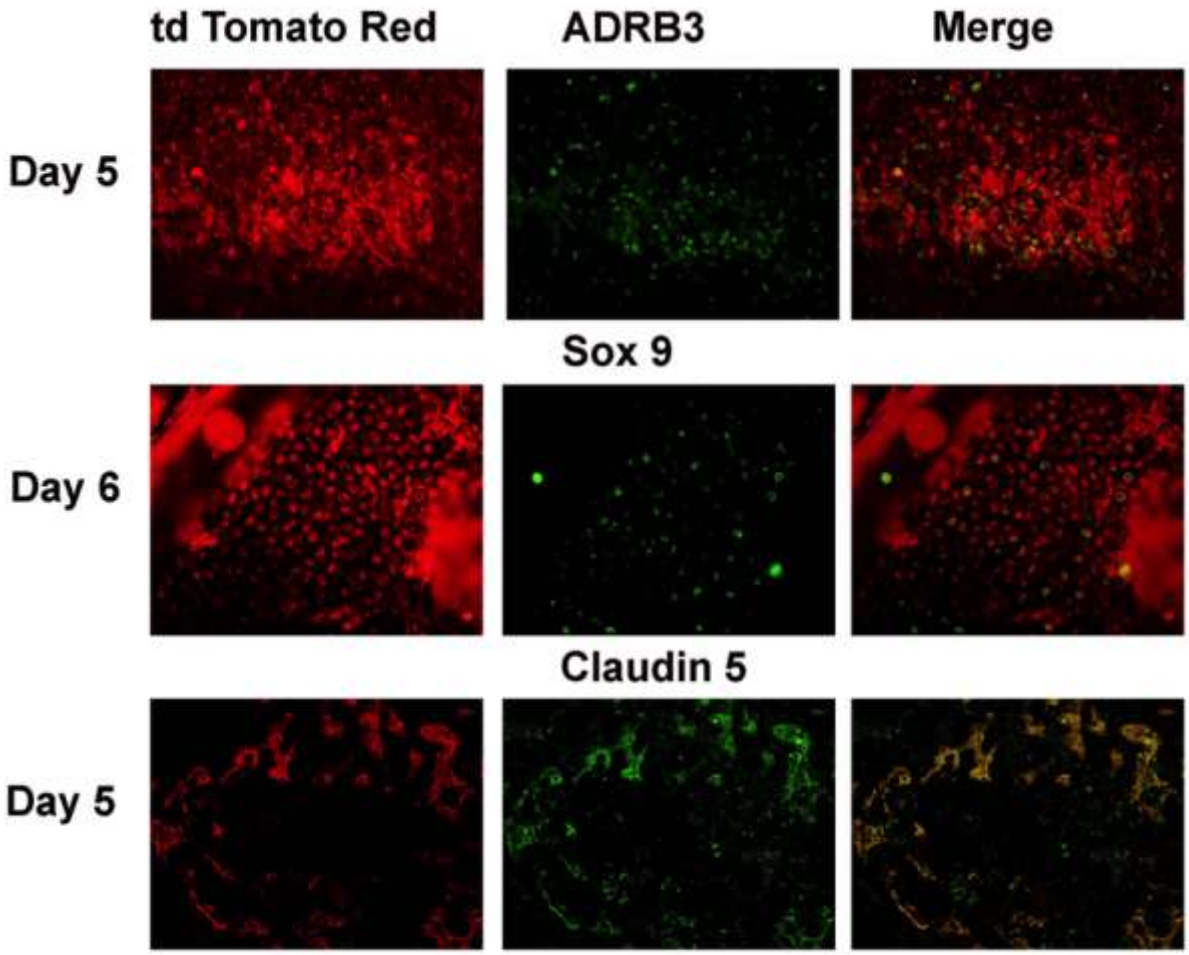


Figure 7. The labeling pattern of pre tBAT and pre-chondrocytes in HO indicate their progenitors arise from nerves. Ert Wnt 1 Cre x R26R td Tomato red mice (n=6) were treated with tamoxifen on days -2, -1, and 0 and then injected with 5 x 10^6 BMP2 producing cells on day

0. On days 5 or 6 the mice were euthanized, the limbs around the injection site harvested and serial frozen sections prepared in sucrose to maintain the fluorescence of td Tomato red.

Task 3: Identification of key changes in the blood profiles that may represent a potential biomarker for HO. In this area we have found that a definite biomarker for HO is MMP9, which we have previously published and are now suggesting be tested clinically [Azhdarinia et al., 2011] [Rodenberg et al., 2011]. However, another biomarker we find that circulates in blood early after the induction of HO is claudin 5 [Lazard et al., 2015]. Our Cooperative Research and Development Agreement with the US Department of the Navy is now in place (J. Forsberg, T. Davis) and we hope to analyze human blood to confirm this analysis in mice.

KEY RESEARCH ACCOMPLISHMENTS:

- Confirmation of the osterix expression in cells within the endoneurium of peripheral nerves.
- Demonstration that the osterix+ cells also express the unique tight junction molecule claudin 5.
- Demonstration that the osterix+ claudin 5+ cells also express PDGFRα, which is a neural marker not associated with normal blood vasculature.
- Demonstrated that suppression of nerve remodeling through delivery of cromolyn, did not suppress the expansion of the claudin 5+ cells; although we have previously shown that this drug suppresses bone formation, through suppression of perineurial cell expansion.
- Demonstrated the rapid increase in Wnt1 during heterotopic ossification, which appeared to co-express claudin 5. This strongly indicates that the osteoprogenitors are formed from neural progenitors or neural stem cells.
- *Successfully implemented a lineage tracing transgenic mouse strain (Ert Wnt1Cre x R26R td tomato red). Showed that Wnt1 was specifically induced only in the presence of tamoxifen and BMP2. Showed that td tomato red-tagged cells expressed either osterix (osteoprogenitors), Sox9 (chondroprogenitors), or brown adipocyte progenitor (β3 adrenergic receptor) markers indicating that the nerve-derived Wnt1 positive (td tomato red positive) cell is at least multipotent, if not pluripotent.*
- Found a marker (claudin 5) early in HO in the mouse model that appears in the blood. We will test for this marker in the blood of humans for correlation to HO. If positive a diagnostic blood test could be developed for HO.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

Salisbury E.A., Lazard Z.W., Ubogu E.E., Olmsted-Davis E.A., Davis A.R. Astrocyte-like cells from the peripheral nerve generate brown adipocytes and contribute to endochondral bone formation. International Bone and Mineral Society 43rd International Sun Valley Workshop: Musculoskeletal Biology, Sun Valley, ID, Aug 4-7, 2013- oral presentation (Selected for a Young Investigator Award)

Lazard ZW, Olmsted-Davis EA, Salisbury EA, Gugala Z, Sonnet C, Davis EL, Beal E, 2nd, Ubogu EE, Davis AR. 2015. Osteoblasts Have a Neural Origin in Heterotopic Ossification. Clin Orthop Relat Res 473:2790-806.

CONCLUSION: We have identified a cell population in the endoneurium of peripheral nerves that expand during heterotopic ossification (HO). Because of its expression of the unique tight junction molecule claudin 5 we consider this cell to be endothelial-like, but its neural origin is undisputed because of the expression of many neural markers including PDGFα, the neural stem cell marker, musashi, and the low affinity nerve growth factor receptor (p75NTR). We have shown that these cells rapidly expand 10-fold within 48 hours of induction of HO, and then start to decrease by 4 days after induction through fluorescence activated cell sorting. Osterix expression was analyzed in the isolated claudin 5+ and – populations and results demonstrated that osterix expression co-purified with the claudin 5+ population. We further, have shown that these cells also express the previously reported osteogenic marker PDGFRα, which is not expressed on normal blood vasculature, but associated with the nervous system. We found that the expansion was not suppressed by cromolyn, which we previously have shown suppressed expansion of perineruial progenitors. In studies not described in detail in this report, we found that these cells traversed the blood-nerve barrier, most likely by their expression of claudin 5, entered the endoneurial vasculature, and flowed through the vasculature to the site of bone formation where they extravasated into the newly forming bone [Lazard et al., 2015]

REFERENCES:

Alfieri KA, Forsberg JA, Potter BK. 2012. Blast injuries and heterotopic ossification. Bone Joint Res 1:192-7.
Argaw AT, Gurfein BT, Zhang Y, Zameer A, John GR. 2009. VEGF-mediated disruption of endothelial CLN-5 promotes blood-brain barrier breakdown. Proc Natl Acad Sci U S A 106:1977-82.
Arpornmaeklong P, Brown SE, Wang Z, Krebsbach PH. 2009. Phenotypic characterization, osteoblastic differentiation, and bone regeneration capacity of human embryonic stem cell-derived mesenchymal stem cells. Stem Cells Dev 18:955-68.
Azhdarinia A, Wilganowski N, Robinson H, Ghosh P, Kwon S, Lazard ZW, Davis AR, Olmsted-Davis E, Sevick-Muraca EM. 2011. Characterization of chemical, radiochemical and optical properties of a dual-labeled MMP-9 targeting peptide. Bioorg Med Chem 19:3769-76.
Gherghe CM, Duan J, Gong J, Rojas M, Klauber-Demore N, Majesky M, Deb A. 2011. Wnt1 is a proangiogenic molecule, enhances human endothelial progenitor function, and increases blood flow to ischemic limbs in a HGF-dependent manner. FASEB J 25:1836-43.

Gonzalez DM, Medici D. 2014. Signaling mechanisms of the epithelial-mesenchymal transition. Sci Signal 7:re8.
Lazard ZW, Olmsted-Davis EA, Salisbury EA, Gugala Z, Sonnet C, Davis EL, Beal E, 2nd, Ubogu EE, Davis AR. 2015. Osteoblasts Have a Neural Origin in Heterotopic Ossification. Clin Orthop Relat Res 473:2790-806.
Lounev VY, Ramachandran R, Wosczyna MN, Yamamoto M, Maidment AD, Shore EM, Glaser DL, Goldhamer DJ, Kaplan FS. 2009. Identification of progenitor cells that contribute to heterotopic skeletogenesis. J Bone Joint Surg Am 91:652-63.
Medici D, Shore EM, Lounev VY, Kaplan FS, Kalluri R, Olsen BR. 2010. Conversion of vascular endothelial cells into multipotent stem-like cells. Nat Med 16:1400-6.
Morita K, Sasaki H, Furuse M, Tsukita S. 1999. Endothelial claudin: claudin-5/TMVCF constitutes tight junction strands in endothelial cells. J Cell Biol 147:185-94.
Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, Das SR, de Ferranti S, Despres JP, Fullerton HJ, Howard VJ, Huffman MD, Isasi CR, Jimenez MC, Judd SE, Kissela BM, Lichtman JH, Lisabeth LD, Liu S, Mackey RH, Magid DJ, McGuire DK, Mohler ER, 3rd, Moy CS, Muntner P, Mussolino ME, Nasir K, Neumar RW, Nichol G, Palaniappan L, Pandey DK, Reeves MJ, Rodriguez CJ, Rosamond W, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Woo D, Yeh RW, Turner MB. 2015. Heart Disease and Stroke Statistics-2016 Update: A Report From the American Heart Association. Circulation.
Nitta T, Hata M, Gotoh S, Seo Y, Sasaki H, Hashimoto N, Furuse M, Tsukita S. 2003. Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. J Cell Biol 161:653-60.
Olmsted-Davis E, Gannon FH, Ozen M, Ittmann MM, Gugala Z, Hipp JA, Moran KM, Fouletier-Dilling CM, Schumara-Martin S, Lindsey RW, Heggeness MH, Brenner MK, Davis AR. 2007. Hypoxic adipocytes pattern early heterotopic bone formation. Am J Pathol 170:620-32.
Piera-Velazquez S, Hawkins DF, Whitecavage MK, Colter DC, Stokes DG, Jimenez SA. 2007. Regulation of the human SOX9 promoter by Sp1 and CREB. Exp Cell Res 313:1069-79.
Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. 1999. Multilineage potential of adult human mesenchymal stem cells. Science 284:143-7.
Rodenberg E, Azhdarinia A, Lazard ZW, Hall M, Kwon SK, Wilganowski N, Salisbury EA, Merched-Sauvage M, Olmsted-Davis EA, Sevick-Muraca EM, Davis AR. 2011. Matrix metalloproteinase-9 is a diagnostic marker of heterotopic ossification in a murine model. Tissue Eng Part A 17:2487-96.
Salisbury E, Hipp J, Olmsted-Davis EA, Davis AR, Heggeness MH, Gannon FH. 2012a. Histologic identification of brown adipose and peripheral nerve involvement in human atherosclerotic vessels. Hum Pathol 43:2213-22.
Salisbury E, Rodenberg E, Sonnet C, Hipp J, Gannon FH, Vadakkan TJ, Dickinson ME, Olmsted-Davis EA, Davis AR. 2011. Sensory nerve induced inflammation contributes to heterotopic ossification. J Cell Biochem 112:2748-58.
Salisbury EA, Lazard ZW, Ubogu EE, Davis AR, Olmsted-Davis EA. 2012b. Transient brown adipocyte-like cells derive from peripheral nerve progenitors in response to bone morphogenetic protein 2. Stem Cells Transl Med 1:874-85.
Wang Y, Li YP, Paulson C, Shao JZ, Zhang X, Wu M, Chen W. 2014. Wnt and the Wnt signaling pathway in bone development and disease. Front Biosci (Landmark Ed) 19:379-407.

Wosczyna MN, Biswas AA, Cogswell CA, Goldhamer DJ. 2012. Multipotent progenitors resident in the skeletal muscle interstitium exhibit robust BMP-dependent osteogenic activity and mediate heterotopic ossification. J Bone Miner Res 27:1004-17.
Yosef N, Ubogu EE. 2013. An immortalized human blood-nerve barrier endothelial cell line for in vitro permeability studies. Cell Mol Neurobiol 33:175-86.
Yosef N, Xia RH, Ubogu EE. 2010. Development and characterization of a novel human in vitro blood-nerve barrier model using primary endoneurial endothelial cells. J Neuropathol Exp Neurol 69:82-97.
Zhang Y, Zhang J, Navrazhina K, Argaw AT, Zameer A, Gurfein BT, Brosnan CF, John GR. 2010. TGFbeta1 induces Jagged1 expression in astrocytes via ALK5 and Smad3 and regulates the balance between oligodendrocyte progenitor proliferation and differentiation. Glia 58:964-74.